

# Generation of Transgenic Dairy Cattle from Transgene-Analyzed and Sexed Embryos Produced *In Vitro*

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We have generated a transgenic calf from *in vitro* produced bovine embryos which had undergone transgene analysis and sexing prior to the embryo transfer. Bovine oocytes were isolated from slaughterhouse-derived ovaries, matured and fertilized *in vitro* and subsequently microinjected with a *dam*-methylated gene construct consisting of genomic sequences encoding human erythropoietin and governed by bovine  $\alpha$ S1-casein regulatory sequences. After 6 to 7 days in culture, the embryos were biopsied and while the embryo remained in culture, the biopsy was subjected to transgene analysis and sexing. The transgene analysis was accomplished with a combined treatment of the embryo lysates with DpnI restriction endonuclease and Bal31 exonuclease followed by polymerase chain reaction (PCR). The transgene analysis was based on the fact that DpnI only cleaves its recognition sequence if the adenine in the sequence is methylated. Pregnancy was induced by the transfer of three viable female embryos with a distinct transgene signal to a hormonally synchronized heifer recipient. Amniotic fluid analysis performed two months after the embryo transfer confirmed the presence of the transgene. The calf born was found to be transgenic by PCR analysis from blood, ear and fetal membranes. The presence of the transgene was also confirmed by Southern blotting.

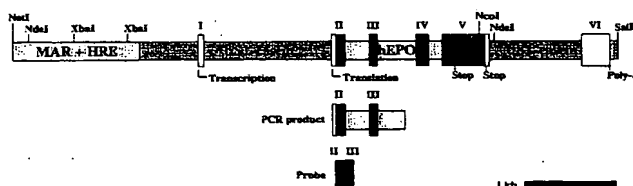
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With the advent of transgenic technology, the production of valuable proteins of pharmaceutical or industrial interest in large farm animals has become an attractive alternative to microbial and animal cell bioreactors. During the past few years, several transgenic domestic animals with targeted transgene expression have been created. These include transgenic sheep producing high quantities of human  $\alpha$ 1-antitrypsin in the milk<sup>1</sup>, transgenic goats expressing in their mammary gland human tissue plasminogen activator<sup>2</sup> and transgenic dairy cattle harboring a human lactoferrin gene governed by bovine  $\alpha$ S1-casein regulatory sequences<sup>3</sup>. Similarly, a successful generation of transgenic swine with high level expression of human protein C in their milk has recently been reported<sup>4</sup>. The production of substantial amounts of human hemoglobin has been accomplished with the aid of transgenic pigs, in which the human hemoglobin level in the circulation accounted for up to 9% of the total hemoglobin<sup>5</sup>. The generation of transgenic farm animals is, however, prohibitively expensive because of the long gestation period, small litter size and high maintenance costs of these animals. The common use of *in vivo* matured embryos likewise requires large number of donor animals and makes the embryo supply limited. In the case of dairy cattle, the latter has been overcome by using *in vitro* matured and fertilized bovine oocytes which have been subsequently cultured *in vitro* to a stage suitable for embryo transfers<sup>6</sup>. However, even with this practically unlimited supply of embryos, because of the very low transgenesis rate in farm animals, a large number of recipients is required for embryo transfers. This bottleneck could be simply solved by a reliable screen of the embryos for transgene integration prior to transfer. Several attempts have been made to establish such a transgene detection system for the embryos of large domestic animals<sup>6,7</sup>. The use of direct PCR with transgene-targeted primers for embryo selection has been shown to yield a high number of false positives<sup>6,7</sup>. Better selection of true transgene-positive embryos can be accomplished by the use of *dam* methylated gene constructs for micro-injections and DpnI digestion of embryo biopsies prior to the PCR analysis<sup>8</sup>.

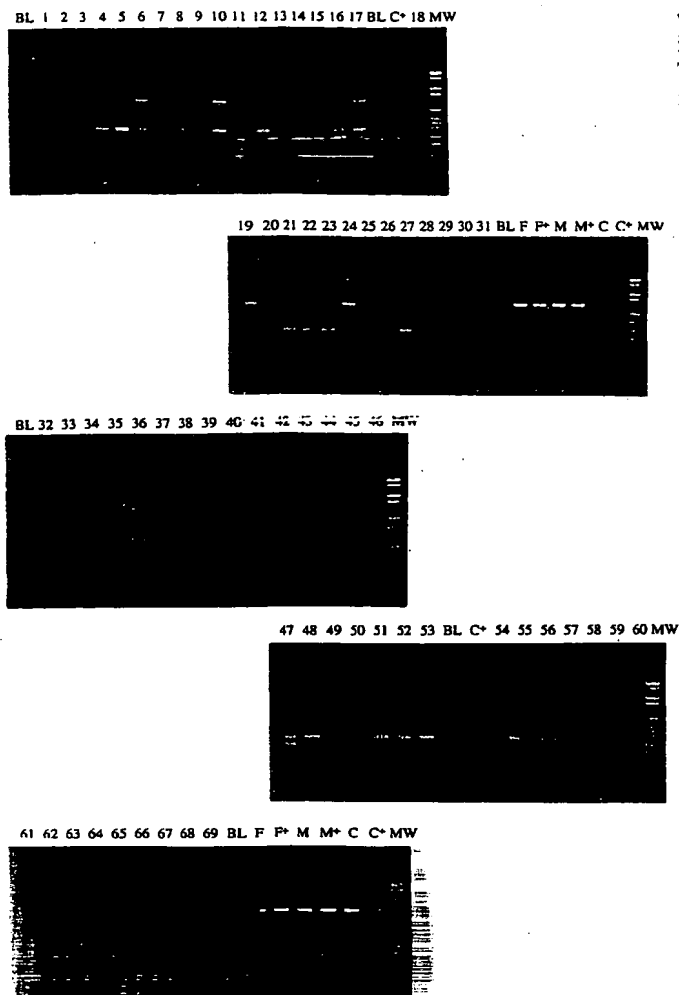
We now report the successful generation of transgenic dairy cattle developed from *in vitro* cultured embryos which have been screened for transgene and sex prior to embryo transfer.

## Results

**Production and analysis of bovine embryos.** After *in vitro* maturation and fertilization (as described<sup>9</sup>), the bovine zygotes were microinjected with a gene construct consisting of bovine  $\alpha$ S1-casein promoter and polyadenylation signal sequences combined with the genomic sequences of the human erythropoietin gene. Chicken lysozyme A element coupled to mouse mammary tumor virus hormone response element was cloned 5' to the bovine  $\alpha$ S1-casein regulatory sequences (Fig. 1). Altogether 1198 *in vitro* fertilized zygotes were produced on two consecutive days and 859 (71%) of them contained visible pronuclei and were microinjected. For the sexing and transgene analysis, 82 morulae and blastocysts were biopsied (9.5% of the injected zygotes), and 69 biopsies were analyzed. The internal control signal was visible in 57 biopsies and 7 embryos (embryos 6, 7, 10, 17, 19, 24 and 36) displayed distinct transgene-signals (12% transgenesis) (Fig. 2). Besides distinct



**FIGURE 1.** The structure of the bovine casein-human erythropoietin (AHRECASEPO) gene construct. White boxes represent casein and black boxes erythropoietin exons. MAR, chicken lysozyme A element 1.3 kb HindIII-XbaI fragment<sup>12</sup>; HRE, mouse mammary tumour virus hormone response element (bases 7112-7295 from pMSG, Pharmacia, Sweden);  $\alpha$ S1-casein, bovine  $\alpha$ S1-casein regulatory sequences bases -610-1483 and 16301-17665 (ref. 13); hEPO, human erythropoietin coding sequences bases 1269-2961 (ref. 14). The AHRECASEPO specific PCR product is 819 bp in size and the probe hybridizes to 3.9 kb NcoI-Xba and 5.1 kb NdeI fragments.



**FIGURE 2.** Analysis of microinjected bovine blastocysts and compact morulas with digestion-PCR. Lysed embryonic biopsies (samples 1-69) were digested with DpnI-Bal31 mixture before amplification with the primers 695 (5'-TTACCTGTTCTGTGGCTGTTGCT-3'), 220 (5'-GTGCAGTGGTGTGATCA-CAGCT-3'), 458 (5'-TGGACCCAGGCAAAGAGACTAG-3'), 337 (5'-CACGCTGCAATTCCAATACACAGAG-3') and 338 (5'-CAAGC-TAATCGATCCATCCTATAGTC-3'). Primers 695 and 220 amplify a 819 bp product from the transgene, primers 695 and 458 amplify a 370 bp product from bovine  $\alpha$ S1-casein and primers 337 and 338 amplify a 287 bp product from bovine Y-chromosome. BL, lysate buffer; C, microinjection DNA (12 fg); F, female bovine DNA (70 pg) mixed with transgenic mouse DNA (140 pg); M, male bovine DNA (70 pg) mixed with transgenic mouse DNA (140 pg); MW, molecular weight markers (pBR328 BglI + pBR328 HinfI). The control samples (C, F and M) were analyzed with (+) and without DpnI-Bal31 digestion.

transgene-signals, 5 questionable signals (Fig. 2, embryos 2, 9, 34, 41 and 57) were detected in the analysis. Thereafter, only viable embryos with a distinct transgene-signal were transferred to two hormonally synchronized heifer recipients, one receiving one male embryo and the other three female embryos.

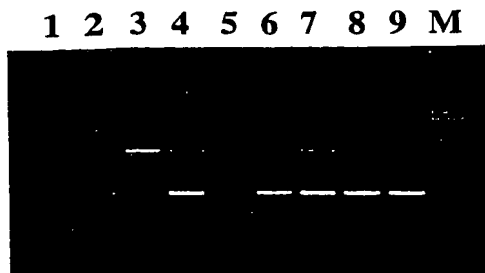
**Generation of transgenic calf.** Pregnancy was achieved with the heifer receiving female embryos (Fig. 2, embryos 6, 7 and 36) and the presence of the transgene was confirmed in the amniotic fluid analysis (Fig. 3) after two months. All the analyzed samples from the subsequently born calf were transgene-positive by PCR (Fig. 3) and also by Southern analysis (Fig. 4). The estimated transgene copy number in the calf was one, and because the transgene locus contained only one copy, the calf

was probably not mosaic. The weaker signal in Figure 4 track 3 is likely due to the lesser amount of DNA in the sample. The animal was healthy and the hematocrit was in the normal range (38%).

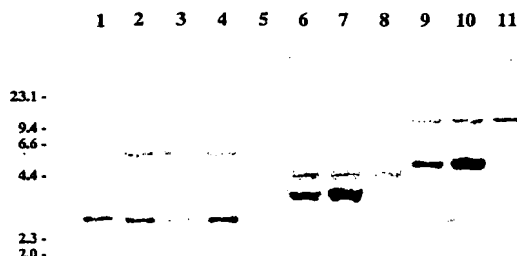
**Reliability of the screening assay.** Between October 1992 and November 1993 we microinjected and analyzed about 10,000 fertilized oocytes, and with transferable embryos were able to produce three calves plus one established pregnancy. The transgenesis rate among analyzed embryos varied substantially between experiments, from 2.6 to 35.7%, average being 15.4%. The first two pregnancies were produced with one viable embryo, one giving a questionable (visible, but very weak) transgene signal and the other a somewhat stronger signal by *in vitro* analysis. Subsequent analysis of the amniotic fluid samples of these fetuses showed that they were transgene-negative as were the born calves. We have since confirmed that biopsied embryos giving questionable PCR signals are, for the most part, transgene negative. The third pregnancy, resulting from the transfer of embryos with distinct transgene-signals, produced the transgenic calf.

## Discussion

The production of transgenic farm animals in general and transgenic dairy cattle in particular is extremely costly and labor-intensive, mainly due to the long gestation period, small litter size, low transgene integration rate and technical difficulties related to the embryo manipulations. Some of the difficulties, such as the limited availability of fertilized bovine oocytes, can be overcome to some extent by maturing and fertilizing slaughterhouse-derived oocytes *in vitro*, as we and some others<sup>1</sup> have done. However, even with the unlimited availability of bovine zygotes, compromises have to be made with regard to the low development rate of *in vitro* produced embryos. As a random transfer of embryos developed from microinjected zygotes requires a large number of recipient animals owing to the low transgene integration rate, a reliable screening of the embryos for transgene integration prior to transfer would be important. Direct PCR analysis with primers targeted to the transgene construct, however, gives unacceptable high rates of false positives<sup>6,7</sup>. And our own results have indicated that more than 70% of microinjected bovine blastocysts; and 30-70% of uninjected control blastocysts exposed to the microinjection DNA in the injection chamber are transgene-positive according to direct PCR analysis. Only control blastocysts which had never been exposed to exogenous DNA at any stage were consistently PCR negative (unpublished). Even the inclusion of DpnI digestion of the *dam* methylated gene construct prior to PCR analysis is not reliable enough in our experience. As utilized here, the inclusion of both DpnI and Bal31 exonuclease in the assay system appears to give the best results, providing that the digestion conditions have been properly determined for each individual gene construct. As biopsying *in vitro*-produced bovine embryos decreases embryonic viability to some extent, the low viability can be compensated by transferring multiple embryos to each recipient. In that case, however, it is essential to sex the embryos and transfer only embryos of the same sex to one recipient in order to avoid freemartinism. In practice, sexing of preimplantation bovine embryos in conjunction with the transgene analysis requires no additional efforts, provided that the primers are compatible with each other. The inclusion of  $\alpha$ S1-casein sequences as internal controls makes the analysis more reliable as unsuccessful assays due to lost samples, biopsies of poor quality and problems in PCR are not judged as false negatives. The large variation in transgenesis rate between experiments may be attributable to the differences in *in vitro* development of embryos and to the different persons doing microinjections. Generally, the poorer the embryonic development after microin-



**FIGURE 3.** PCR analysis of amniotic fluid sample and tissues of the transgenic calf. The controls (1-3 and 5) and calf samples (4, 6-9) were amplified for 32 cycles with the primers 695, 220, 458, 337 and 338. 1, Female bovine DNA (1 ng); 2, male bovine DNA (1 ng); 3, transgenic mouse DNA (10 ng, one AHRECA-SEPO copy/cell); 4, amniotic fluid (60  $\mu$ l) lysate; 5, lysate buffer; 6, blood (0.75  $\mu$ l) lysate; 7, ear lysate; 8 and 9, placenta lysate; MW, molecular weight markers (pBR328 BglI + pBR328 HinfI). The transgene specific PCR product is 819 bp, the bovine  $\alpha$ -S1-casein specific PCR product is 370 bp and the bovine Y-chromosome specific PCR product is 287 bp in size.



**FIGURE 4.** Southern blot analysis of the transgenic calf. DNA (15  $\mu$ g) were prepared from the transgenic calf's placenta (2), ear (3) and blood (4, 6 and 9) and from the blood of a non-transgenic cow (5, 8 and 11). Transgenic mouse (one AHRECA-SEPO copy/cell) DNA (1) and non-transgenic cow DNA mixed with 30 pg microinjection DNA (7 and 10) were used as gene copy number controls. After digestion with EcoRI (1-5), NcoI + XbaI (6-8) and NdeI (9-11), the samples were electrophoresed, transferred to positively charged Nylon membrane and hybridized with digoxigenin labeled single stranded PCR probe. The probe was detected using Lumigen PPD as a substrate. Transgene specific hybridization products are 2.8 kb (1-5), 3.9 kb (6-8) and 5.1 kb (9-11) in size.

jection, the higher is the transgenesis rate.

By initially transferring gene-injected, whole embryos into 30 recipients we obtained 17 pregnancies. However, none of the resulting calves was transgenic. The good pregnancy efficiency but the lack of transgenic offspring may perhaps be explained by the fact that only the fastest developing, best quality embryos were transferred. Our later observations have indicated that the transgenesis frequency among the fastest developing embryos is less than 5% according to the screen employed here (unpublished observation). The frequency of successful pregnancies dropped significantly after we started to transfer only biopsied, transgene-positive embryos; so far 27 transfers have resulted in only 4 pregnancies. At present, the absolute reliability of our *in vitro* screen is impossible to judge. However, even with a successful screening of preimplantation bovine embryos for transgene integration and sex, thousands of microinjections have to be carried out to obtain a single pregnancy with a transgene-positive embryo.

### Experimental Protocol

**Production of microinjected, transgene analyzed and sexed bovine embryos.** A detailed protocol is described elsewhere<sup>9</sup>. Briefly, oocytes were collected from slaughterhouse ovaries, matured and fertilized with frozen-thawed bull semen. The zygotes were vortexed, centrifuged and pronucleus-injected with the AHRECA-SEPO-gene construct (2  $\mu$ g/ml) into one of the pronuclei. The zygotes were subsequently cultured in 50- $\mu$ l co-culture drops with bovine oviductal epithelial cells<sup>10</sup>. Compact moru-

lae and blastocysts were biopsied 7 to 8 days after fertilization with a microblade under stereomicroscope by hand. The biopsies, representing about one third of the embryo, were collected into lysate buffer tubes, whereas the remaining embryos were cultured until embryo transfers. Lysed biopsies were digested with DpnI (30 mU) - Bal31 (100  $\mu$ U) mixture before PCR amplification with transgene,  $\alpha$ -S1-casein and Y-chromosome specific primers for 35 cycles. The results, the presence of the transgene and the embryonic sex, were visualized after agarose gel electrophoresis of the PCR products.

**Embryo transfers and amniocentesis.** After overnight culture viable transgene positive embryos were transferred non-surgically to the uterine horn ipsilateral to the corpus luteum of the recipients, which had been hormonally synchronized with two cloprostenol injections (Estrumate<sup>®</sup>, 0.5 mg/recipient) twelve days apart. The amniotic samples were aseptically aspirated from the amniotic cavity through a flank incision under local anaesthesia at two months of pregnancy.

**DNA preparation and analysis.** PCR analyses from amniotic fluid and calf samples were carried out using lysates made in PCR-buffer containing proteinase K (200  $\mu$ g/ml). Ear and placental tissue was used directly, but blood samples (50  $\mu$ l) were diluted 1:10 with 10 mM-Tris 1 mM-EDTA pH 8.0 buffer and centrifuged, whereas amniotic fluid samples (2 ml) were centrifuged, before suspending into lysate buffer. The samples were amplified for 32 cycles in 50  $\mu$ l volume directly after proteinase K digestion (2 h + 60°C). For Southern blot analysis, genomic DNA was prepared with proteinase K digestion and phenol/chloroform extraction from placenta, ear and blood. After restriction enzyme digestions, the samples were electrophoresed on 0.9% agarose gels and transferred to positively charged Nylon membranes (Boehringer Mannheim) using capillary transfer. A single stranded digoxigenin-labeled probe (Fig. 1) was prepared with PCR, hybridizations and subsequent chemiluminescence detections using Lumigen PPD (Boehringer Mannheim) as a substrate were done as recently described<sup>11</sup>.

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